PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classificati n 6 : C12N 15/29, 15/52, 15/82, A01H 5/00 | A1

(11) International Publication Number: **WO 97/21816**

(43) International Publicati n Date: 19 June 1997 (19.06.97)

(21) International Application Number: PCT/GB96/03076

(22) International Filing Date: 12 December 1996 (12.12.96)

(30) Priority Data:
9525459.5 13 December 1995 (13.12.95) GB

(71) Applicant *(for all designated States except US)*: ZENECA LIMITED [GB/GB]; 15 Stanhope Gate, London W1Y 6LN (GB).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: MANNING, Kenneth [GB/GB]; Horticulture Research International, Wellesbourne, Warwick CV35 9EF (GB).

(74) Agents: HUSKISSON, Frank, Mackie et al.; ZENECA Agrochemicals, Intellectual Property Dept., Jealott's Hill Research Station, P.O. Box 3538, Bracknell, Berkshire RG42 6YA (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: GENETIC CONTROL OF FRUIT RIPENING

(57) Abstract

The ripening characteristics of strawberries are modified by genetic transformation with one or more than one ripening-related DNA selected from Sequences 1 to 9.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

GENETIC CONTROL OF FRUIT RIPENING

This invention relates generally to the modification of a plant phenotype by the regulation of plant gene expression. More specifically it relates to the control of fruit ripening by control of one or more than one gene which is known to be implicated in that process.

Two principal methods for the control of expression are known. These are referred to in the art as "antisense downregulation" and "sense downregulation" or "cosuppression". Both of these methods lead to an inhibition of expression of the target gene. Overexpression is achieved by insertion of one or more than one extra copies of the selected gene. Other lesser used methods involve modification of the genetic control elements, the promoter and control sequences, to achieve greater or lesser expression of an inserted gene.

In antisense downregulation, a DNA which is complementary to all or part of the target gene is inserted into the genome in reverse orientation and without its translation initiation signal. The simplest theory is that such an antisense gene, which is transcribable but not translatable, produces mRNA which is complementary in sequence to mRNA product transcribed from the endogenous gene: that antisense mRNA then binds with the naturally produced "sense" mRNA to form a duplex which inhibits translation of the natural mRNA to protein. It is not necessary that the inserted antisense gene be equal in length to the endogenous gene sequence: a fragment is sufficient. The size of the fragment does not appear to be particularly important. Fragments as small as 40 or so nucleotides have been reported to be effective. Generally somewhere in the region of 50 nucleotides is accepted as sufficient to obtain the inhibitory effect. However, it has to be said that fewer nucleotides may very well work: a greater number, up to the equivalent of full length, will certainly work. It is usual simply to use a fragment length for which there is a convenient restriction enzyme cleavage site somewhere downstream of fifty nucleotides. The fact that only a fragment of the gene is required means that not all of the gene need be sequenced. It also means that commonly a cDNA will suffice, obviating the need to isolate the full genomic sequence.

The antisense fragment does not have to be precisely the same as the endogenous complementary strand of the target gene. There simply has to be sufficient sequence similarity to achieve inhibition of the target gene. This is an important feature of antisense technology as it permits the use of a sequence which has been derived from one plant species to be effective in another and obviates the need to construct antisense vectors for each individual species of interest. Although sequences isolated from one species may be effective in another, it is not infrequent to find exceptions where the degree of sequence similarity between one species and the other is insufficient for the effect to be obtained. In such cases, it may be necessary to isolate the species-specific homologue.

Antisense downregulation technology is well-established in the art. It is the subject of several textbooks and many hundreds of journal publications. The principal patent reference is European Patent No. 240,208 in the name of Calgene Inc. There is no reason to doubt the operability of antisense technology. It is well-established, used routinely in laboratories around the world and products in which it is used are on the market.

Both overexpression and downregulation are achieved by "sense" technology. If a full length copy of the target gene is inserted into the genome then a range of phenotypes is obtained, some overexpressing the target gene, some underexpressing. A population of plants produced by this method may then be screened and individual phenotypes isolated. As with antisense, the inserted sequence is lacking in a translation initiation signal. Another similarity with antisense is that the inserted sequence need not be a full length copy. Indeed, it has been found that the distribution of over- and under- expressing phenotypes is skewed in favour of underexpression and this is advantageous when gene inhibition is the desired effect. For overexpression, it is preferable that the inserted copy gene retain its translation initiation codon. The principal patent reference on cosuppression is European Patent 465,572 in the name of DNA Plant Technology Inc. There is no reason to doubt the operability of sense/cosuppression technology. It is well-established, used routinely in laboratories around the world and products in which it is used are on the market.

Sense and antisense gene regulation is reviewed by Bird and Ray in Biotechnology and Genetic Engineering Reviews 9: 207-227 (1991). The use of these techniques to control selected genes in tomato has been descibed by Gray et.al., Plant Molecular Biology, 19: 69-87 (1992) and is described herein to control the expression of selected genes in strawberries.

Gene control by any of the methods described requires insertion of the sense or antisense sequence, with appropriate promoters and termination sequences containing polyadenylation signals, into the genome of the target plant species by transformation, followed by regeneration of the transformants into whole plants. It is probably fair to say that transformation methods exist for most plant species or can be obtained by adaptation of avaliable methods.

For dicotyledonous plants the most widely used method is *Agrobacterium*- mediated transformation. This is the best known, most widely studied and, therefore, best understood of all transformation methods. The rhizobacterium *Agrobacterium* tumefaciens, or the related *Agrobacterium* rhizogenes, contain certain plasmids which, in nature, cause the formation of disease symptoms, crown gall or hairy root tumours, in plants which are infected by the bacterium. Part of the mechanism employed by Agrobacterium in pathogenesis is that a section of plasmid DNA which is bounded by right and left border regions is transferred stably into the genome of the infected plant. Therefore, if foreign DNA is inserted into the so-called "transfer" region (T-region) in substitution for the genes normally present therein, that foreign gene will be transferred into the plant genome. There are many hundreds of references in the journal literature, in textbooks and in patents and the methodology is well-established.

The effectiveness of *Agrobacterium* is restricted to the host range of the microorganism and is thus restricted more or less to dicotyledonous plant species. In general monocotyledonous species, which include the important cereal crops, are not amenable to transformation by the *Agrobacterium* method. Various methods for the direct insertion of DNA into the nucleus of monocot cells are known.

In the ballistic method, microparticles of dense material, usually gold or tungsten, are fired at high velocity at the target cells where they penetrate the cells, opening an

aperture in the cell wall through which DNA may enter. The DNA may be coated on to the microparticles or may be added to the culture medium.

In microinjection, the DNA is inserted by injection into individual cells via an ultrafine hollow needle.

Another method, applicable to both monocots and dicots, involves creating a suspension of the target cells in a liquid, adding microscopic needle-like material, such as silicon carbide or silicon nitride "whiskers", and agitating so that the cells and whiskers collide and DNA present in the liquid enters the cell.

In summary, then, the requirements for both sense and antisense technology are known and the methods by which the required sequences may be introduced are known. What remains, then is to identify genes whose regulation will be expected to have a desired effect, isolate them or isolate a fragment of sufficiently effective length, construct a chimeric gene in which the effective fragment is inserted between promoter and termination signals, and insert the construct into cells of the target plant species by transformation. Whole plants may then be regenerated from the transformed cells.

This invention is concerned with the control of ripening in fruit, and the particular interest here is in strawberries.

The interest in controlling the ripening process is to improve the flavour and/or texture of the fruit both characters being largely affected by the ripening process. Sugars are the most important soluble component of the flavour. Some 99% of the soluble sugars in strawberry is accounted for by sucrose, glucose and fructose, the amount of these sugars being affected by the season but their relative proportions are largely unaffected.

There is little information in the literature on the metabolic pathways involved in the synthesis of sugars in strawberry. It is known, however that sugars are synthesised during the ripening of the fruit.

The changes in gene expression during strawberry fruit ripening and their regulation by auxin have been described in Planta 194: 62-68 (1994)

An object of the present invention is to provide DNA sequences enabling the construction of DNAs suitable for the control of ripening in strawberries.

According to the present invention there is provided a vector for use in the genetic transformation of strawberry cells in order to regulate ripening, comprising a promoter sequence, a regulation sequence and a transcription termination sequence, in which the regulation sequence is selected from the group consisting of Sequences 1 through 9 given herein.

In a variation of the vector of this invention the regulation sequence varies from Sequences 1 through 9 but retains sufficient similarity to be effective in gene regulation. Thus, the regulatory gene may be a homologue of a gene of sequence 1 through 9 which has been obtained from a different plant species.

The gene regulation sequence may be in the same or antisense orientation as the endogenous target gene. It may also be a of partial or full sequence length. The invention further contemplates the overexpression of one or more of the genes represented by the DNAs provided by inserting into the strawberry genome one or more than one extra copies thereof.

The invention also provides a gene regulation sequence selected from Sequences 1 through 9 herewith and sequences which are obtainable from said sequences by the use thereof as probes.

Promoters suitable for use in constructs of the invention may be any sutable promoters which are known to be effective in driving expression of foreign genes in plants, for example the promoters may be those which are isolatable from the genomic version of the cDNAs of the invention.

The invention also provides a strawberry plant and propagating material thereof which contains a vector of this invention.

Further according to the invention, there is provided a method for the control of ripening of strawberry fruit comprising inserting into the genome of the cell of a strawberry plant a gene regulation vector aforesaid.

The invention further provides genetically improved strawberry plants which ripen more slowly that their unaltered counterparts.

The gene regulation sequences of the invention may be synthesised from the sequence information given or may be isolated from a library. To assist isolation we have

deposited with the National Collection of Industrial & Marine Bacteria, St. Machar Drive, Aberdeen, UK, a cDNA library of strawberry ripening genes. The library was deposited on 15th November 1994 and has the Accession Number NCIMB 40693.

Thus, this invention is based on the identification of genes which encode proteins involved in strawberry ripening-related processes. DNA sequences which encode these proteins have been cloned and characterised. The DNA sequences may be used to modify plant ripening characteristics of fruit.

By virtue of this invention strawberry plants can be generated which, amongst other phenotypic modifications, may have one or more of the following fruit characteristics:

improved resistance to damage during harvest, packaging and transportation due to slowing of the ripening and over-ripening processes;

longer shelf life and better storage characteristics due to reduced activity of degradative pathways (e.g. cell wall hydrolysis);

improved processing characteristics due to changed activity of proteins/enzymes contributing to factors such as: viscosity, solids, pH, elasticity;

improved flavour and aroma at the point of sale due to modification of the sugar/acid balance and other flavour and aroma components responsible for characteristics of the ripe fruit;

modified colour due to changes in activity of enzymes involved in the pathways of pigment biosynthesis (e.g. lycopene, β-carotene, chalcones and anthocyanins);

increased resistance to post-harvest pathogens such as fungi.

The activity of the ripening-related proteins may be either increased or reduced depending on the characteristics desired for the modified plant part (fruit, leaf, flower, etc). The levels of protein may be increased; for example, by incorporation of additional genes. The additional genes may be designed to give either the same or different spatial and temporal patterns of expression in the fruit. "Antisense" or "partial sense" or other techniques may be used to reduce the expression of ripening-related protein.

The activity of each ripening-related protein or enzyme may be modified either individually or in combination with modification of the activity of one or more other

ripening- related proteins/enzymes. In addition, the activities of the ripening-related proteins/enzymes may be modified in combination with modification of the activity of other enzymes involved in fruit ripening or related processes.

DNA constructs according to the invention may comprise a base sequence at least 10 bases (preferably at least 35 bases) in length for transcription into RNA. There is no theoretical upper limit to the base sequence - it may be as long as the relevant mRNA produced by the cell - but for convenience it will generally be found suitable to use sequences between 100 and 1000 bases in length. The preparation of such constructs is described in more detail below.

As a source of the DNA base sequence for transcription, a suitable cDNA or genomic DNA or synthetic polynucleotide may be used. The isolation of suitable ripening-related sequences is described above; it is convenient to use DNA sequences derived from the ripening-related clones deposited at NCIMB in Aberdeen. Sequences coding for the whole, or substantially the whole, of the appropriate ripening-related protein may thus be obtained. Suitable lengths of this DNA sequence may be cut out for use by means of restriction enzymes. When using genomic DNA as the source of a base sequence for transcription it is possible to use either intron or exon regions or a combination of both.

To obtain constructs suitable for expression of the appropriate ripening-related sequence in plant cells, the cDNA sequence as found in one of the strawberry plasmids or the gene sequence as found in the chromosome of the strawberry plant may be used. Recombinant DNA constructs may be made using standard techniques. For example, the DNA sequence for transcription may be obtained by treating a vector containing said sequence with restriction enzymes to cut out the appropriate segment. The DNA sequence for transcription may also be generated by annealing and ligating synthetic oligonucleotides or by using synthetic oligonucleotides in a polymerase chain reaction (PCR) to give suitable restriction sites at each end. The DNA sequence is then cloned into a vector containing upstream promoter and downstream terminator sequences. If antisense DNA is required, the cloning is carried out so that the cut DNA sequence is inverted with respect to its orientation in the strand from which it was cut.

In a construct expressing antisense RNA, the strand that was formerly the template strand becomes the coding strand, and vice versa. The construct will thus encode RNA

in a base sequence which is complementary to part or all of the sequence of the ripening-related mRNA. Thus the two RNA strands are complementary not only in their base sequence but also in their orientations (5' to 3').

In a construct expressing sense RNA, the template and coding strands retain the assignments and orientations of the original plant gene. Constructs expressing sense RNA encode RNA with a base sequence which is homologous to part or all of the sequence of the mRNA. In constructs which express the functional ripening-related protein, the whole of the coding region of the gene is linked to transcriptional control sequences capable of expression in plants.

For example, constructs according to the present invention may be made as follows. A suitable vector containing the desired base sequence for transcription is treated with restriction enzymes to cut the sequence out. The DNA strand so obtained is cloned (if desired, in reverse orientation) into a second vector containing the desired promoter sequence and the desired terminator sequence. Suitable promoters include the 35S cauliflower mosaic virus promoter, the polyubiquitin promoter and the tomato polygalacturonase gene promoter sequence (Bird et al, 1988, Plant Molecular Biology, 11:651-662) or other developmetally regulated fruit promoters. Suitable terminator sequences include that of the *Agrobacterium tumefaciens* nopaline synthase gene (the nos 3' end).

The transcriptional initiation region (or promoter) operative in plants may be a constitutive promoter (such as the 35S cauliflower mosaic virus promoter) or an inducible or developmentally regulated promoter (such as fruit-specific promoters), as circumstances require. For example, it may be desirable to modify ripening-related protein activity only during fruit development and/or ripening. Use of a constitutive promoter will tend to affect ripening-related protein levels and functions in all parts of the plant, while use of a tissue specific promoter allows more selective control of gene expression and affected functions. Thus in applying the invention it may be found convenient to use a promoter that will give expression during fruit development and/or ripening. Thus the antisense or sense RNA is produced only in the organ in which its action is required and/or only at the time required. Fruit development and/or ripening-specific promoters that could be used include the ripening-enhanced polygacturonase

promoter (International Patent Publication Number WO92/08798), the E8 promoter (Diekman & Fischer, 1988, EMBO, 7:3315-3320), the fruit specific 2A11 promoter (Pear et al, 1989, Plant Molecular Biology, 13:639-651), the histidine decarboxylase promoter (HDC, Sibia) and the phytoene synthase promoter.

Ripening-related protein or enzyme activity (and hence ripening-related processes and fruit ripening characteristics) may be modified to a greater or lesser extent by controlling the degree of the appropriate ripening-related protein's sense or antisense mRNA production in the plant cells. This may be done by suitable choice of promoter sequences, or by selecting the number of copies or the site of integration of the DNA sequences that are introduced into the plant genome. For example, the DNA construct may include more than one DNA sequence encoding the ripening-related protein or more than one recombinant construct may be transformed into each plant cell.

The activity of each ripening-related protein may be separately modified by transformation with a suitable DNA construct comprising a ripening-related sequence. In addition, the activity of two or more ripening-related proteins may be simultaneously modified by transforming a cell with two or more separate constructs. Alternatively, a plant cell may be transformed with a single DNA construct comprising both a first ripening-related sequence and a second ripening-related sequence.

It is also possible to modify the activity of the ripening-related protein(s) while also modifying the activity of one or more other enzymes. The other enzymes may be involved in cell wall metabolism or in fruit development and ripening. Cell wall metabolising enzymes that may be modified in combination with a ripening-related protein include but are not limited to: pectin esterase, polygalacturonase, β-galactanase, β-glucanase. Other enzymes involved in fruit development and ripening that may be modified in combination with a ripening-related protein include but are not limited to: ethylene biosynthetic enzymes, carotenoid biosynthetic enzymes including phytoene synthase, carbohydrate metabolism enzymes.

Several methods are available for modification of the activity of the ripening-related protein(s) in combination with other enzymes. For example, a first plant may be individually transformed with a ripening-related gene construct and then crossed with a second plant which has been individually transformed with a construct encoding another

enzyme. As a further example, plants may be either consecutively or co-transformed with ripening-related constructs and with appropriate constructs for modification of the activity of the other enzyme(s). An alternative example is plant transformation with a ripening-related construct which itself contains an additional gene for modification of the activity of the other enzyme(s). The ripening-related gene constructs may contain sequences of DNA for regulation of the expression of the other enzyme(s) located adjacent to the ripening-related sequences. These additional sequences may be in either sense or antisense orientation as described in International patent application publication number WO93/23551 (single construct having distinct DNA regions homologous to different target genes). By using such methods, the benefits of modifying the activity of the ripening-related proteins may be combined with the benefits of modifying the activity of other enzymes.

A DNA construct of the invention is transformed into a target plant cell. The target plant cell may be part of a whole plant or may be an isolated cell or part of a tissue which may be regenerated into a whole plant. For any particular plant cell, the ripening-related sequence used in the transformation construct may be derived from the same plant species, or may be derived from any other plant species (as there will be sufficient sequence similarity to allow modification of related isoenzyme gene expression).

Transgenic plants and their progeny may be used in standard breeding programmes, resulting in improved plant lines having the desired characteristics. For example, fruit-bearing plants expressing a ripening-related construct according to the invention may be incorporated into a breeding programme to alter fruit-ripening characteristics and/or fruit quality. Such altered fruit may be easily derived from elite lines which already possess a range of advantageous traits after a substantial breeding programme: these elite lines may be further improved by modifying the expression of a single targeted ripening-related protein/enzyme to give the fruit a specific desired property.

By transforming plants with DNA constructs according to the invention, it is possible to produce plants having an altered (increased or reduced) level of expression of one or more ripening-related proteins, resulting from the presencein the plant genome of DNA capable of generating sense or antisense RNA homologous or complementary to the RNA that generates such ripening-related proteins. For fruit-bearing plants, fruit may be

obtained by growing and cropping using conventional methods. Seeds may be obtained from such fruit by conventional methods (for example, tomato seeds are separated from the pulp of the ripe fruit and dried, following which they may be stored for one or more seasons). Fertile seed derived from the genetically modified fruit may be grown to produce further similar modified plants and fruit.

The fruit derived from genetically modified plants and their progeny may be sold for immediate consumption, raw or cooked, or processed by canning or conversion to soup, sauce or paste. Equally, they may be used to provide seeds according to the invention.

The genetically modified plants (transformed plants and their progeny) may be heterozygous for the ripening-related DNA constructs. The seeds obtained from self fertilisation of such plants are a population in which the DNA constructs behave like single Mendelian genes and are distributed according to Mendelian principles: eg, where such a plant contains only one copy of the construct, 25% of the seeds contain two copies of the construct, 50% contain one copy and 25% contain no copy at all. Thus not all the offspring of selfed plants produce fruit and seeds according to the present invention, and those which do may themselves be either heterozygous or homozygous for the defining trait. It is convenient to maintain a stock of seed which is homozygous for the ripening-related DNA construct. All crosses of such seed stock will contain at least one copy of the construct, and self-fertilized progeny will contain two copies, i.e. be homozygous in respect of the character. Such homozygous seed stock may be conventionally used as one parent in F1 crosses to produce heterozygous seed for marketing. Such seed, and fruit derived from it, form further aspects of our invention. We further provide a method of producing F1 hybrid plants expressing a ripening-related DNA sequence which comprises crossing two parent lines, at least one of which is homozygous for a ripening-related DNA construct. A process of producing F1 hybrid seed comprises producing a plant capable of bearing genetically modified fruit homozygous for a ripening-related DNA construct, crossing such a plant with a second homozygous variety, and recovering F1 hybrid seed. It is possible according to our invention to transform two or more plants with different ripening-related DNA constructs and to cross the progeny of the resulting lines, so as to obtain seed of plants which contain two or more constructs leading to reduced expression of two or more fruit-ripening-related proteins.

The invention will now be described, by way of illustration, by the following Examples and with reference to the following figures in which:

Figure 1 shows a diagrammatic map of plasmid pBINCEL, derived from pBINPLUS.

Figure 2 shows the results of agarose gel analysis of

1. pBINCEL - plasmid construct with antisense cellulase PCR fragment using primers from 35S promoter and 5' to 3' cellulase.

2. genomic DNA from transformed strawberry PCR fragment using primers from 35S promoter and 5' to 3' cellulase.

Figure 3 shows the results of a northern blot analysis of O-methyl transferase, chalcone synthase, flavanoid-3-hydroxylase, UDP glucosyl flavonol transferase and UDP glucuronosyl transferase gene expression in wild type strawberries.

Figure 4 shows the results of a northern blot analysis of invertase gene expression in wild type strawberries.

EXAMPLE 1

Construction of a cDNA library of ripening genes

1.1 Isolation of messenger RNA

Total mRNA was isolated from ripe fruit tissue (the receptacle with the achenes removed) of strawberry (Fragaria x ananassa Duch. cv. Brighton) as described by Manning K. Analytical Biochemistry 195, 45-50 (1991). Messenger RNA was isolated from total RNA by oligo(dT)-cellulose chromatography according to Bantle et.al., Analytical Biochemistry 72, 413-427 (1976).

1.2 Synthesis of cDNA

The first and second strands of the cDNAs were synthesised from messenger RNAs using a commercial cDNA synthesis kit (RPN.1256Y: Amersham Life Sciences, Amersham, Bucks., UK), priming the first strand cDNA synthesis with olgio-dT.

1.3 Cloning into vector

Double stranded cDNAs were cloned into the λgt10 vector using the BRL cloning system (8287SA: Bethseda Research Laboratories, Paisley, Renfrewshire, UK) essentially as follows. Internal EcoR1 sites of the cDNAs were methylated using EcoR1 methylase. The DNA termini were repaired with T4 DNA polymerase and phosphorylated EcoR1 linkers ligated to the cDNA woth T4 ligase. Excess linkers were digested and removed by column chromatography on DEAE-Sephadex. The purified double stranded cDNAs with EcoR1 termini were ligated into λgt10 vector DNA digested with EcoR1 and dephosphorylated. Vector DNA was then packaged using an in vitro packaging extract (Promega Corporatiom, Southampton, UK). Recombinant bacteriophage were mixed with plating bacteria (E.coli C600 hflA 150) as decribed in the BRL protocol to determine titre, for library screening and subsequent amplification.

1.4 Screening of the cDNA library from ripe strawberry

The unamplified cDNA library from ripe strawberry was differentially screened using cDNA from fruit receptacle tissue at the ripe and white stages of ripeness. A proportion of the library was plated at low density and duplicate plaque lifts made on to Hybond N nylon filters (Amersham) according to the manufacturer's instructions. One filter was hybridised to ripe cDNA from white fruit and the duplicate filter hybridised to ripe cDNA. Hybridisations were at high stringency using digoxigenin as a non-radioactive label (Boehringer Mannheim, Lewes, Sussex, UK). Plaques hybridising preferentially to ripe cDNA were picked and replated at low density for a second round of selection by differential screening. Single plaques from the second screening were picked and numbered as ripening-enhanced clones.

1.5 Characterisation of the ripe cDNA library and ripening-enhanced clones

The ripe cDNA library was prepared with an efficiency of 3.03×10^6 plaque-forming units per microgram of cDNA. The size of the cDNA inserts in this library ranged from approximately 0.24 to 6 kbp with a mean insert size of approximately 1.4 kbp.

From the 343 plaques used in the first screen, 83 putative ripening clones were obtained. Of these, 48 were pure clones with single inserts, the remainder being impure and having multiple inserts.

The 48 clones with single inserts were partially sequenced using the DyeDeoxy (Trade Mark) Terminator Cycle Sequencing Kit (Applied Biosystems, Warrington, Cheshire, UK) with forward and reverse primers specific for the λgt_{10} vector. From these, the following nine ripening-related clones were selected. Comparison of these sequences in the EMBL database using GCG ('Wisconsin') software has identified homologies for the clones listed in Table I.

TABLE I

Sequence-ID-No	Clone identity/Accession	Clone number(s)	Approx. size (kbp)
1	Chalcone synthase (type 2)	60,100	1.45
2	Flavanone-3-hydroxylase (type 1)	2,50,68,84,88	1.35
3	Flavanone-3-hydroxylase (type 2)	85	1.7
4	Flavanone-3-hydroxylase (type 3)	89	1.35
5	Flavanone-3-hydroxylase (type 4)	55a	1.0
6	UDP-glucose glucosyl-transferase	74	1.1
7	UDP-glucuronosyl transferase (ERT 1b)	109	1.05
8	Invertase	21	1.915
9	Invertase	27	1.553

Clones 2, 50, 55a, 68, 84, 85, 88 and 89 are members of the same gene family, with clones 2, 50, 68 and 88 being identical and clones 55a, 85, and 89 representing three other genes in this family.

EXAMPLE 2

Construction of antisense RNA vectors with the CaMV35S promoter

A vector is constructed using the sequences corresponding to a fragment of the insert of one of the sequences 1 to 9. This fragment is synthesised by polymerase chain reaction using synthetic primers. The ends of the fragment are made flush with T4 polymerase and it is cloned into the vector pJR1 which has previously been cut with SmaI. pJR1 (Smith et al, 1988, Nature, 334:724-726) is a Bin19 (Bevan, 1984, Nucleic Acids Research, 12:8711-8721) based vector, which permits the expression of the antisense RNA under the control of the CaMV 35S promoter. This vector includes a nopaline synthase (nos) 3' end termination sequence.

Alternatively a vector is constructed using a restriction fragment obtained from a strawberry ripening-related clone which is then cloned into the vectors GA643 (An et al, 1988, Plant Molecular Biology Manual A3: 1-19) or pDH51 (Pietrzak et al, 1986, Nucleic Acids Research, 14:5875-5869) which has previously been cut with a compatible restriction enzyme(s). A restriction fragment from the ripening related sequence/pDH51 clone containing the promoter, the sequence of interest and other pDH51 sequence is cloned into SLJ44026B or SLJ44024B (Jones et al, 1990, Transgenic Research, 1) or Bin19 (Bevan, 1984, Nucleic Acids Research, 12:8711-8721) which permits the expression of the antisense RNA under control of the CaMV 35S promoter. This procedure is illustrated in Figures 2 and 3.

After synthesis of the vector, the structure and orientation of the sequences are confirmed by DNA sequence analysis.

EXAMPLE 3

Construction of antisense RNA vectors with a fruit enhanced promoter.

The fragment of the ripening-related cDNA that was described in Example 2 is also cloned into the vector pJR3. pJR3 is a Bin19 based vector, which permits the expression of the antisense RNA under the control of the tomato polygalacturonase (PG) promoter. This vector includes approximately 5 kb of promoter sequence and 1.8 kb of 3' sequence from the PG promoter separated by a multiple cloning site.

After synthesis, vectors with the correct orientation of the ripening-related sequences are identified by DNA sequence analysis.

Alternative fruit enhanced promoters (such as E8 or 2A11) are substituted for the polygalacturonase promoter in pJR3 to give alternative patterns of expression.

EXAMPLE 4

Construction of truncated sense RNA vectors with the CaMV 35S promoter

The fragment of the ripening-related cDNA that was described in Example 2 is also cloned into the vectors described in Example 2 in the sense orientation.

After synthesis, the vectors with the sense orientation of the phytoene synthase sequence are identified by DNA sequence analysis.

EXAMPLE 5

Construction of truncated sense RNA vectors with fruit-enhanced promoter.

The fragment of the ripening-related cDNA that was described in Example 3 is also cloned into the vectors described in Example 3 in the sense orientation.

After synthesis, the vectors with the sense orientation of the ripening-related sequence are identified by DNA sequence analysis.

EXAMPLE 6

Construction of an over-expression vector using the CaMV35S promoter

The complete sequence of a ripening-related cDNA containing a full open-reading frame is inserted into the vectors described in Example 2.

EXAMPLE 7

Construction f an ver-expression vector using a fruit-enhanced promoter

The complete sequence of a ripening-related cDNA containing a full open-reading frame is inserted into the vectors described in Example 3 (pJR3 or alternatives with different promoters).

EXAMPLE 8

Generation of transformed plants

Vectors are transferred to *Agrobacterium tumefaciens* LBA4404 (a micro-organism widely available to plant biotechnologists) and are used to transform strawberry plants. Transformation follows standard protocols (e.g. Bird et al. 1988, Plant Molecular Biology. 11:651-662). Transformed plants are identified by their ability to grow on media containing the antibiotic kanamycin. Plants are regenerated and grown to maturity. Ripening fruit are analyzed for modifications to their ripening characteristics.

The transformation of strawberry, for example to control selected genes, may also be carried out as follows. The sequence of a near full length cDNA from strawberry encoding the enzyme cellulase was inserted in the antisense orientation as described in Example 2 into a pBINPLUS vector (van Engelen et al (1995) Transgenic Research 4, 288-290) containing the cauliflower mosaic virus (CaMV) 35S promoter-nos3' terminator cassette from pJR1Ri inserted into the Hind III/ EcoRI site. (pJR1Ri is a derivative of pJR1 (Smith et al (1988) Nature 334 724-26) which is made by substituting a HindIII/SstII fragment containing the wild type nos/nptII cassette from pGA472 for the equivalent fragment in pJR1 and then inverting the EcoRI/HindIII CaMV35S/nos3' fragment using linkers. A map of pJR1Ri is described in published International Patent Application No. WO 94/03619). Strawberry (cv Calypso) leaf discs were transformed by coincubation with the kanamycin sensitive *Agrobacterium tumefaciens* strain EHA105 (a strain widely available to plant biotechnologists and described in Hood et al, Transgenic Research 2 208-218 (1995)) containing the pBINPLUS antisense construct. Explants were grown on regeneration medium initially containing 100mg/ml kanamycin. After three weeks the explants were transferred to regeneration medium without kanamycin. At 4-6 weeks putatively transformed shoots were cultured on propagation medium for two weeks then transformants selected on medium containing 25mg/l kanamycin.

Transformation of strawberry with other desired genes may be achieved in an analogous manner to that described above for transformation with cDNA encoding cellulase

EXAMPLE 9

Evidence of Successful Transformation

A 1400bp PCR fragment obtained from genomic DNA from a putative strawberry transformant containing an antisense cellulase construct and a similar size PCR fragment obtained from the vector antisense construct used to transform the strawberry were analysed as shown in Figure 2. Figure 1 provides details of the expression vector used in this experiment. The primers used were from the 35S promoter sequence and from the cellulase sequence. The results shown in Figure 2 show that the transgene was incorporated into the strawberry demonstrating successful transformation had been achieved.

EXAMPLE 10

Analysis of Gene Expression During Ripening

Total RNA was extracted from strawberry fruit during normal development and analysed by northern blotting using standard experimental procedures. The results of such an analysis are shown in Figures 3 and 4. The level of mRNA corresponding to the expression of O-methyl transferase, chalcone synthase, flavanoid-3-hydroxylase, UDP-glucosyl flavonol transferase, UDP-glucuronosyl transferase and invertase was monitored in the receptacle at various time points between pollination and the overripe stage. The data provide evidence that O-methyl transferase, chalcone synthase, flavanoid-3-hydroxylase, UDP-glucosyl flavonol transferase, UDP-glucuronosyl transferase and invertase are involved in the ripening process in normal fruit development.

SEQ-ID-NO-1
Clone: 060
Identity: Chalcone synthase (type 2)
060.seq Length: 524 July 17, 1995 15:12 Type: N Check: 1359 ..

1 GGGTCCGGTC ACCGTTCTTG GCCATCGGGA CCGCAACTCC TCCCAACTGT

51 ATTGACCAGA GCACGTACCC CGACTACTAC TTTCGTNTCA CCAACAGCGA

101 GCACAAGGCT GAGCTCANGG AGNAAATTCC AGCGTCATGT GTGACAAATC

151 TATGATCAAG AAGCGTTACA TGTATTTGAC TGAAGAGATT CTCAAAGGAG

201 CAATCCTAGG CATGTGTGAG TACATGGCAC CTTCACTTGN ATGCAAGACA

251 AGACATGGTG GTGGNTAGNA AATTCCAAAG CTTGGCAAAA GAGGCCGCTG

301 TCAAGGTCAT TAAGGAATGG GGGTCAGNCC AAGTCCAAAA TCACCCACTT

351 GGGTCTTTCG GTACCACTAG TGGTGTCGAC ATNCCCGGTG CCGATTTACC

401 AGCTCACTAA GGCTCTTGGG CCCTCCCGCC CGTCTTTTCA AGNGTCTCAN

451 NAATGTTCCC AGCAANGGNT GTTTTCGGCC CGNAGGGNAC GGGGNCTCCN

501 GNTTGGNAAA AGGGTCTTGG CCCA

SEQ-ID-NO-2
Clone: 084
Identity: Flavanone 3-hydroxylase (type 1)
084.seq Length: 507 July 17, 1995 15:24 Type: N Check: 9579 ..

1 AAAAATTCTC AGGCAGATCG CTAGAGAGCA TATATCAGAA TGGNCCCTAC

51 TCCTACTACT CTGACCGTCA TAGTGGGGGA GAAGACCCTC CAACAGAGCT

101 TCGTCCGCGA CGNAAGTATG AGCGCCCTAA GGTGGCCTAC AACCAATTCA

151 GNAATGATAT TCCGATCATT TCCCTCTCTG GCATCGAAGA GGTCGAAGGC

201 CGCCGCGCTG AGATTTGCAA GAAGATTGTT GAGGCCTGCG AGGNACTGGG

251 GCGTTTTCCA GATTGTTGAT CACGGTTATC GTACCCCAAG CTCATCTCGG

301 AAATGACTCG TCTCGCCAGA GGAGTTCTTC GNTTTGNCGC CGGAGGGAAA

351 AGCTCCGCTT TCGACATTTC CCCGNGGCAA AAAAGGGTNG CTTCATCGNT

401 TTCCAGCCAT TTACAGNGAG ANGCGGTNCA GNATTGGTGC GAGATTGTNN

451 ACCTACTTCT CATACCCGGN GGNGCCACCC AGACTNCTCG AGGTNGNCCN

501 TATANAN

SEQ-ID-NO-3
Clone: 085
Identity: Flavanone 3-hydroxylase (type 2)
085.Seq Length: 486 July 17, 1995 15:33 Type: N Check: 7729 ..

1 CTTAGCCAAA GCCGGAATCG ATTATCATGC ATCCAACTAC CTATTTATTT

51 GGTTACACTA CTGATTCTAT ATAAACACTG CTGCTAGGTC TAAAGCTTCC

101 ATCATTAAAA GCATAACGTA CAACAAGCCC TAAGAAGCTT TTGTAAGTAG

151 TGTACGTAGA GAGATCGAAA GAGAGAGCTA TAGCTAGAAG CGACAATGGT

201 GACTGCTGCA TCCATTGGTT CAAGAGTCGA GAGTTTGGCC AGCAGCGGGA

251 TCTCAACGAT CCCAAAGGAG TACGTGAGAC CCGAAGAGGA GCTCGTTAAC

301 ATCGGTGACA TCTTCGAAGA CGAGAAGAGC ACCGAAGGGN CTCAAGTACC

351 TACCATTGAT TTGAGGGAGA TAGACTCGGN GGACATCAAG GTGAGGGAGA

401 TTTGGAGGNT TTTNGGNNGA NACCAGCCCN CGNCTGGGGT TNATGAACCT

451 NGNCACCNTG GAACTCCNNG GNGTCATGAC GGGTCA

SEQ-ID-NO-4
Clone: 089
Identity: Flavanone 3-hydroxylase (type 3)
089.seq Length: 510 July 17, 1995 15:31 Type: N Check: 2979 ..

1 TTTTGGAATA CACCGCCTAA CAATGGCTGN AGNTCCAAGT GAGTCCATAC

51 CCTCTGTAAA TAAGGCCTGG GTCTATTCAG AGTATGGAAA AACTGCTGAT

101 GTTCTCAAGT CNGATCCAAG TGTGGCTGTT CCTGAAATTA AAGAGGATCA

151 GGTGTCTGAT CAAGGTTGTN GNTGTTTCTC TTAACCCAGT TGNATTTTAA

201 GAGGGNTCTT GGTTACTTCA NGGACACTGA CTCTCCCCTA CCTACAATTC

251 CAGGGTATNA TGTAGCTNGT GTNGCGGTAA AGGTNGGAAG TCAAGTTNAC

301 CANGTTCAAG GTGGNGGATG AAGTGTNTNG GGGATCTCAN CGANACAGNA

351 TTGGTNNACC CAACAANGTN NGGGNCTCTT TGGGCCAGAG NCACACTCNT

401 TNCAGGATTT AAAGAGTTTT TGNCTTACAA AACCCANNTN ACCNCNCANC

451 NTTNNTTGGA AGNATNCTTA GNCNTCCCCC CNGGTTTTTT GTAACTTACC

501 CNCNNAAGGG

SEQ-ID-NO-5
Clone: 055a
Identity: Flavanone 3-hydroxylase (type 4)
055a.seq Length: 559 July 17, 1995 15:38 Type: N Check: 712 ..

1 GGCGGCAAAA TTCAAGGCTA CGGAAGCAAG CTTGCAAACA ATGCTTCCGG

51 GCAACTTGAG TGGGAGGACT ACTTTTTCCA CTGTGTTTAT CCTGAGGACA

101 AGCGTGACTT GTCCATTTGG CCTCAAACAC CTTCCGACTA TATTGTGGCA

151 ACAAGTGAGT ATGCTAAGGA ACTGAGGGGG NTAGCAACCA AGATACTGAG

201 CATACTCTCA CTTGGCTTGG GATTAGAAGA AGGGAGGCTG GAGAAGGAGG

251 TCGGTGGACT CGAAGAACTC CTCATGCAAA TGAAGGATCA NCTACTACCC

301 AAAATGCCCT CAGCCGGAAC TTGCACTCGG CGTGGAAGCT CATACCCGAC

351 ATAAGTGCAC TCACCTTCAT CCTCCACAAN ATGGTTCCCC GNCTGNAGTT

401 CTTCCTACGG GGGNAAATNG NTTGACAGCN AAGGTGNGTC CCCAACTCCG

451 NCGNCATGCA CATNGGCGAC AACCNTAGAG ATTCTTCNGC ACCGGCANTA

501 CAAGAGCATC TTCACAGGGG GGTCCNCCAA CAAGGGGGAA GGCNACGGTC

551 TNCNCTNNC

SEQ-ID-NO-6
Clone: 074
Identity: UDP-glucose glucosyltransferase
Reverse complemented
074.seq Length: 508 July 17, 1995 16:47 Type: N Check: 5861 ..

1 NGGGGAANNC ACTAGTGGGG ATTTCACGNT TAAGGAGGGN AAACCATCTC

51 TGTTNATTTG CNGGAATNNT CGAAAAGTGA AGACCTTCAG GATCCTACNC

101 AGAGGGAATG CATCCTTGCG GGAAAACTTT GGAGTACACT CTNNTAAACG

151 TATNGCTTTC ACNAGAGTGG GGAACTAATT GCTANCCACT TGCAACNCGC

201 AGTTTTNCAT NCAACTCGTT CGAAGAAACT AGACCCTGTG ATCACAAATG

251 ATTTGAAGTC CAAATTNCAA GAGGTTCCTC AACGTGGGAC CATTGGACCT

301 ACTAGAACCA ACAGCGAGTG CAGCCACCAC CACACCGCAG AGCGACGGAA

351 GCTGTTGCCG GAGATGGCTG CTTATCGTGG CTTGATAAAC AGAAGGCGGC

401 GTCCGTGGTC TATGTGAGTT TTGGATCAGT AACAAGACCA TCNCCGGAAG

451 AGCTTATGGC GCTAGCTGAG GCTCTGGAGG CCAGTAGGGT TCCATTCTTG

501 TGGTCACT

SEQ-ID-NO-7
Clone: 109
Identity: ERT 1b (UDP-glucuronosyl transferase)
109.seq Length: 432 July 17, 1995 17:02 Type: N Check: 6063 ..

1 ttgttgagct ccggagtttc tttcatatgg gtgatgaagc ccccacaccc

51 tgattccggc tttgaacttc tggttctgcc ggaagggttt ttggagaagg

101 caggagacag aggcaaagtt gtgcaatgga gtccacaaga gaagattttg

151 gagcatcctt cgacggcttg ctttgtgact cattgcgggt ggaactcaac

201 catggagtca ctcacctcag gaatgcccgt ggtggcattc ccacaatggg

251 gtgaccaagt gaccgacgcc aagtatttgg tcgacgagtt taaggtggga

301 gtaagaatgt gccgnngaga ggncgaagac agggtnnatc cctagggaag

351 aggtagagaa agtncttgnt ggaggngacc tcggnggccc acggnggngg

401 ngatnangna aaacgccttg anattgaagg nt

SEQ-ID-NO-8
Clone: 21
Identity: Invertase

5'-3' sequence

215end.Gcg Length: 1915 July 17, 1995 15:35 Type: N Check: 994 ..

1 cggccatgat gatattctct ctttggcaat tctgccttct ttcacttttg

51 ctttcttttg gtgttattga gcttcaagct tcccaccatg tctatagcaa

101 ccttcaaact acccaacttg cttcaacaca tccccaagct aaagacccct

151 acagaactgg ttatcatttc cagcctcgca agaattggat caatgatcca

201 aatgggccac tgatttacaa gggcatttac catcttttct atcagtacaa

251 tcccagcagt gtagtttggg gtaacattgt ttgggcacat tccacatcca

301 ctgatctcgt caactggatt ccacatgaag ctgctatcta cccatcaatt

351 ctctccgata tcaatggctg ttggtcgggg tccgtcacaa tccttcccag

401 cggaaagccg gccattttat acaccggaat caaccccgac aaagaacaag

451 ttcaaaactt ggcatttcca aaaaaccttt ccgacccatt tcttagggag

501 tgggttaaag tcccacaaaa ccctctaatg gctccaactc aagctaacca

551 aatcaatgcc agctcattta gggatcctac cactgcttgg ctagggccag

601 ataagagatg gaggttgatc attggaagca aaaggaacca caggggacta

651 gctatcctct acagaagcaa agatttcatg cattggacta aggctaaaca

701 tccattatat tcaacgccaa aaaatggtat gtgggaatgc cctgattttt

751 tcccagtttc gaagactaag ttgcttggtc ttgacacatc tgcaattggt

801 ccggatgtta agcatgtact caaagttagc ttggacaaca ctaggaaaga

851 gtactacaca attggtacat ataatgtgag caaggatatc tatatcccag

901 atgatggatc aattgagagt gattctggtt tgagatatga ttatggtaag

951 ttttatgctt caaaaacttt ctttgacagt gctaagaacc gcagaatctt

1001 gtggggttgg atcaatgagt cctcaagtgt tagtggtgac atcaagaaag

1051 gatggtctgg actccaggca attccaagga ctattgtgct cgacaaatct

1101 agaaagcaat tggtgcaatg gcctgtagtg gagcttgaga aacttagaac

1151 aaacgaggtc aagttaccaa gcactctcct taaaggagga tcacttcatg

1201 aagtcattgg tgtcacagca gcacaggctg atgtagatgt tgcatttgag

1251 ataagtgatc tcaagaaagc agaagttatg gatccaagtt ggactaatgc

1301 acaacttttg tgtagtaaaa agggtacctc agtgaaaggg gctctaggac

1351 catttggatt gttggcattt gtttcaaagg atttgaaaga aaagacagca

1401 atcttctata gaattttcaa gtctcacaac aataacaaca aatatgtggt

1451 tcttatgtgc agtgaccaaa gcaggtcttc cctaaaccca gataatgata

1501 tgacaactta tggaacattt gtaaacgtgg atcctcttca tgaaaaattg

1551 tcactaagaa gcttgattga tcactctata gtggagagtt ttggtggaaa

1601 aggcaaggag tgcataacag ctagggtgta tcctacattg gctgttgatg

1651 gtgataccca tttatatgct ttcaattatg gaagtgagag tgtcaaaatc

1701 gcaggaagtg catggagcat gaaaactgct aaaatcaatt gatcaagatt

1751 agaaaagaag tggggaggtt gtattgattc ttgtatgtga cccatctact

1801 tatagtgcct cgtaaattag ataattgata taagagttga ataaacaagt

1851 gtgagccaat tttttgtgct tgcatcaatc cattacgctt gtttttatcc

1901 ggaaaaaaaa aaaaa

SEQ-ID-NO-9
Cl ne: 27
Identity: Invertase
5`-3` sequence
275end.Gcg Length: 1553 July 17, 1995 15:33 Type: N Check: 9419 ..

1 cggtataatg gctccaactc aagctaacca aatcaatgcc agctcattta

51 gggatcctac cactgcttgg ctagggccag ataagagatg gaggttgatc

101 attggaagca aaaggagcca aaggggacta gctatcctct acagaagcaa

151 agatttcatg cattggacta aggctaaaca tccattatat tcaacaccga

201 aaaatggtat gtgggaatgc cctgattttt tcccagtttc gaagactaag

251 ttgcttggtc ttgacacatc tgcaattggt ccggatgtta agcatgtact

301 caaagttagc ttggacaaca ctaggaaaga gtactacaca attggtacat

351 ataatgtgag caaggatatc tatatcccag atgatggatc aattgagagt

401 gattctggtt tgagatatga ttatggtaag ttttatgctt caaaaacctt

451 ctttgacagt gctaagaacc gcagaatctt gtggggttgg atcaatgagt

501 cctcaagtgt tagtggtgac atcaagaaag gatggtctgg actccaggca

551 attccaagga ctattgtgct cgacaaatct ggaaagcaat tggtgcaatg

601 gcctgtagta gagcttgaga aacttagaac aaacgaggtc aagttaccaa

651 gcactctcct taaaggagga tcacttcatg aagtcattgg tgtcacagca

701 gcacaggctg atgtagatgt tgcatttgag ataagtgatc tcaagaaagc

751 agaagttatg gatccaagtt ggactaatgc acaacttttg tgtagtaaaa

801 agggtacctc agtgaaaggg gctctaggac catttggatt gttggcattt

851 gtttcaaagg atttgaaaga aaagacagca atcttctata gaattttcaa

901 gtctcacaac aataacaaca aatatgtggt tcttatgtgc agtgagcaaa

951 gcagatcttc cctaaaccca gataatgata tgacaactta cggagtattt

1001 gtaaatgtgg atcctcttca tgaaaagctg tcattaagaa gtttgattga

1051 tcactctata gtggagagtt ttggtggaaa aggcaaggcg tgcataacag

1101 ctagggtgta tcctacaatg actgttgatg gtgataccca tttatatgca

1151 ttcaattatg gaagtgagag tgtcaaaatc gcaggaagtg catggagcat

1201 gaaaactgct caaatcaatt gatcaagatt agaaaaagaa gtggagaaaa

1251 gaagtgggga ggttgtgttg attattgtat gtgactcatc tacttatggt

1301 gtctcttaaa ttagataatt gatatgagag ttgaataaat aagtgtgagc

1351 caatttttg tgcttgcatc aatccattag gcttgttttt atctgaactt

1401 gtcattgttt gagaatttgt ctaaatgcta gtttggtatt gatgtgactt

1451 taaaaaaaaa ctactgctga tgtacttcaa aaataactag ttgttaatta

1501 aattagtatt gtgtttggta aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa

1551 aaa

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 5, line 27 - 30

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

NCIMB

Address of depositary institution *(including postal code and country)*

St Machar Drive
Aberdeen
United Kingdom

Date of deposit	Accession Number
15 November 1994	40693

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only

☒ This sheet was received with the international application

Authorized officer

CAROL WORTHING
ROOM G.Y73 EXT. 4595

For International Bureau use only

☐ This sheet was received by the International Bureau on:

Authorized officer

29

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION F THE DEPOSIT OF MICROORGAN
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO
Zeneca Seeds,
Jealotts Hill Research Station,
Bracknell, Berkshire.
RG12 6EY

NAME AND ADDRESS
OF DEPOSITOR

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
lambda phage strawberry cDNA library	NCIMB 40693

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[ ] a scientific description

[X] a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 15 November 1994 (date of the original deposit)[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on . (date of receipt of request for conversion)

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name:

Address: 23 St Machar
Aberdeen

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

Date: 18 November 1994

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Form BP/4 (sole page)

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

Zeneca Seeds,
Jealotts Hill Research Station,
Bracknell,
Berkshire.
RG12 6EY

INTERNATIONAL FORM

VIABILITY STATEMENT
Issued pursuant to Rule 10.2 by the INTERNATIONAL DEPOSITARY AUTHORITY identified on the following page

NAME AND ADDRESS OF THE PARTY TO WHOM THE VIABILITY STATEMENT IS ISSUED

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Address: AS ABOVE	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 40693 Date of the deposit or of the transfer: 15 November 1994

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 16 November 1994 [2]. On that date, the said microorganism was

[3] [X] viable

[3] [ ] no longer viable

[1] Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

[2] In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

[3] Mark with a cross the applicable box.

Form BP/9 (first page)

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

INTERNATIONAL DEPOSITARY AUTHORITY

Name: NCIMB Ltd.

Address: 23 St Machar Drive
Aberdeen Scotland
UK AB2 1RY

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

Date: 18 November 1994

[4] Fill in if the information has been requested and if the results of the test were negative.

32

DELIVER TO:

THE NATIONAL COLLECTIONS OF INDUSTRIAL AND MARINE BACTERIA LTD.
23 ST. MACHAR DRIVE
ABERDEEN
AB2 1RY
TEL: (0224) 273412
FAX. (0224) 487658

INVOICE TO:
Zeneca Seeds,
Jealotts Hill Research Station,
Bracknell,
Berkshire.
RG12 6EY
Attention of Mrs. Clare Dowling

VAT No. 381 1905 71

ACCOUNT No.	REFERENCE 1	REFERENCE 2	INVOICE DATE	INVOICE No.	TRANSACTION
	PP/JH 43295DJL	PD2	18.11.94	17059/C	Invoice

PRODUCT	DESCRIPTION	QUANTITY	PRICE	NETT VALUE
	To deposit of one organism for patent purposes	1	£400.00	£400.00

COPY FOR INFORMATION ONLY

PAYMENT SHOULD BE MADE IN BRITISH STERLING TO NCIMB LTD AT THE ABOVE ADDRESS OR DIRECT TO BANK ACCOUNT No. 062404, SORTING CODE 826914, AT THE CLYDESDALE BANK LTD, UNIVERSITY BRANCH, 17 HIGH STREET, OLD ABERDEEN AB2 3EE.

VAT CODE	GOODS	VAT RATE	VAT AMOUNT
	£400.00	17½%	£70.00

INVOICE GOODS	INVOICE VAT	INVOICE TOTAL
£400.00	£70.00	£470.00

Terms: NETT MONTHLY IMPORTANT: Please quote invoice number when making payment.

CLAIMS

1. A vector for use in the genetic transformation of plant cells in order to regulate fruit ripening, comprising a promoter sequence, a regulation sequence and a transcription termination sequence, in which the regulation sequence is selected from the group consisting of Sequences 1 through 9 given herein.

2. A vector for use in the genetic transformation of plant cells in order to regulate fruit ripening, comprising a promoter sequence, a regulation sequence and a transcription termination sequence, in which the regulation sequence has sufficient similarity to any one of Sequences 1 through 9 to be effective in gene regulation.

3. A vector as claimed in claim 1 or claim 2 in which the regulatory gene is an analogue of any one of Sequences 1 through 9 which has been obtained from a different plant species.

4. A vector as claimed in claim 1 or claim 2 or claim 3 in which the gene regulation sequence is in the same or antisense orientation as the endogenous target gene.

5. A vector as claimed in any preceding claim in which the promoter isolated from the genomic equivalent of any of Sequences 1 through 9.

6. A gene regulation sequence selected from Sequences 1 through 9 herewith and sequences which are obtainable from said sequences by the use thereof as probes.

7. A method for the modulation of ripening processes in fruit comprising stably inserting into the genome of a fruit-producing plant one or more copies of a DNA of sequence selected from Sequences 1 through 9 and the genomic equivalents thereof.

8. A method for the modulation of ripening processes in fruit comprising stably inserting into the genome of a fruit-producing plant one or more copies of a DNA of sequence complementary to any one of Sequences 1 through 9, the genomic equivalents thereof and fragments thereof.

9. A method as claimed in claim 7 or claim 8 in which the said plant is a strawberry (Fragaria) plant.

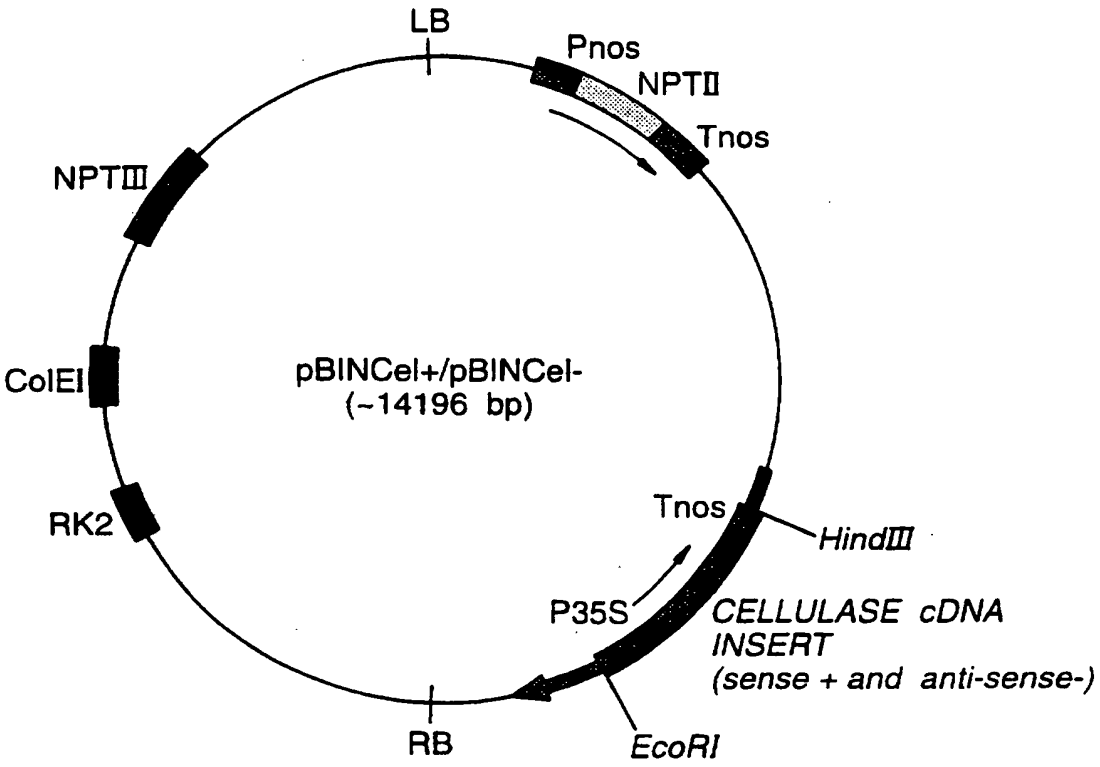
10. A plant and propagating material thereof which contains within its genome a vector of this invention.

11. A strawberry plant and propagating material thereof which contains within its genome a vector of this invention.

12 A genetically modified strawberry plant and propagating material derived therefrom which has a genome comprising a gene expression modulating construct for overexpression or downregulation of an endogenous strawberry plant gene counterpart of Sequences 1 through 9.

13. Each of the gene regulation sequences 1 through 9, isolable from the cDNA library deposited with the National Collection of Industrial & Marine Bacteria, St. Machar Drive, Aberdeen, UK on 15th November 1994 under the Accession Number NCIMB 40693.

Fig.1.



⟸ lacZ

Control construct is as above but without the insert

Constructs are based on pBINPLUS (Trans. Res. 4, 288-290 (1995)

Fig.2.

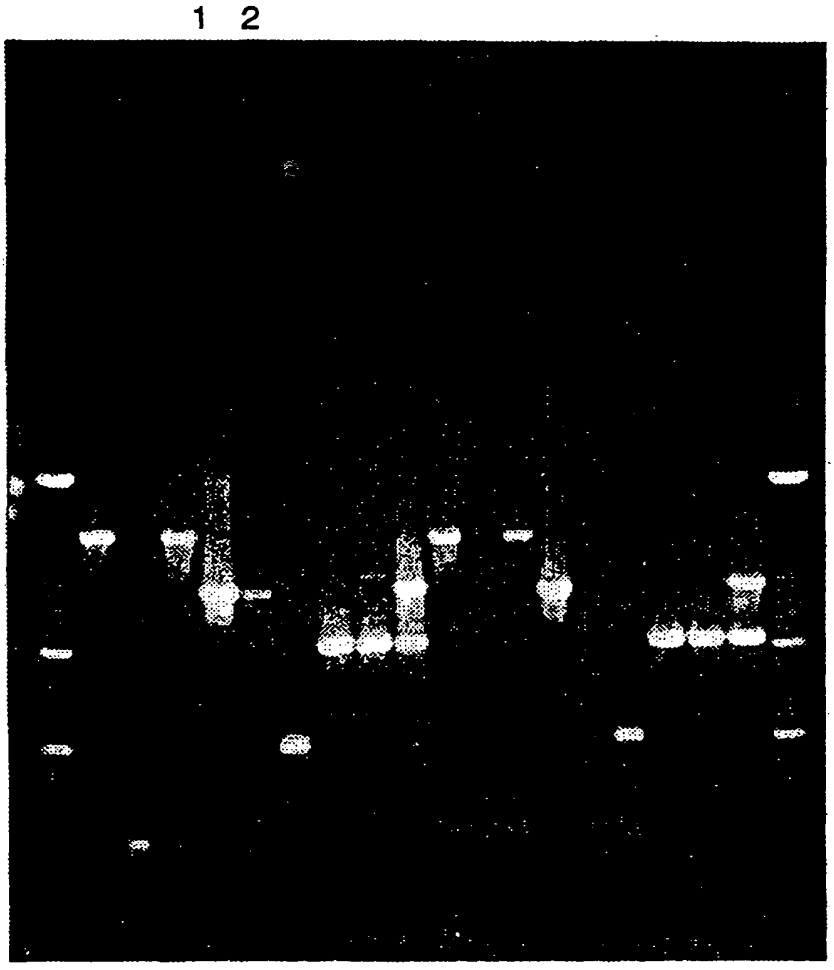


Fig.3.

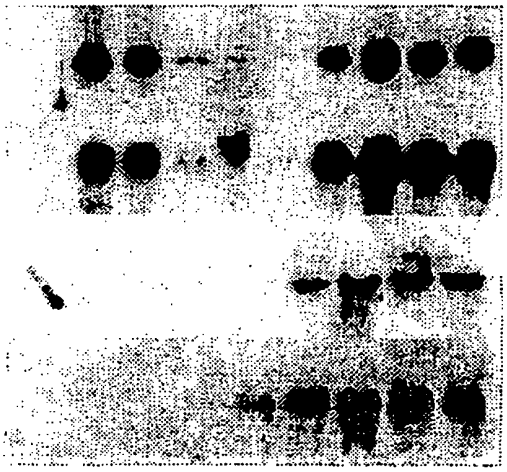
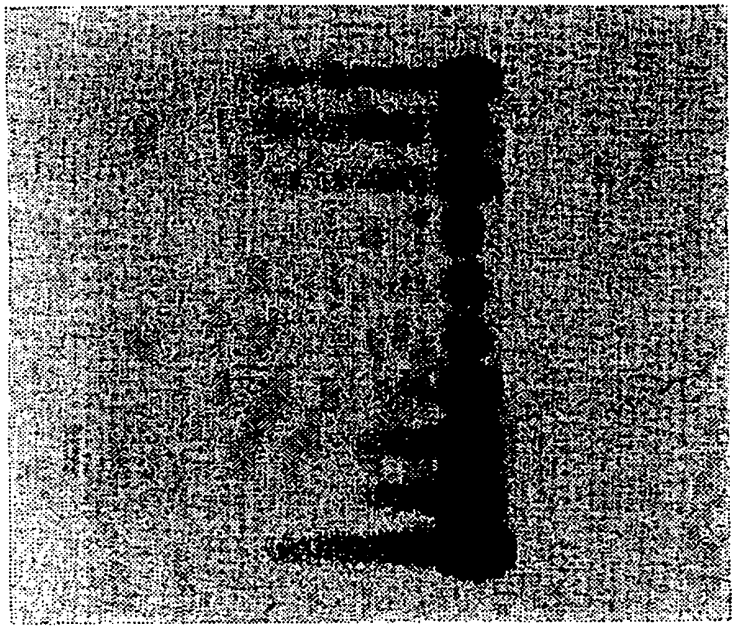

Chalcone synthase
Flavanoid-3-hydroxylase
UDP glucosyl flavonol transferase
UDP glucuronosyl transferase
DAY 4 7 10 13 16 19
turning
orange
ripe
overripe

Fig.4.



INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCI/GB 96/03076

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/29 C12N15/52 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	WO 93 06711 A (UNIV CALIFORNIA) 15 April 1993 see the whole document ---	2-4,7,8, 10 13
X A	WO 92 14831 A (SALK INST BIOTECH IND) 3 September 1992 see the whole document ---	2-4,7,8, 10 13
X	PLANT JOURNAL, vol. 3, no. 3, 1993, pages 469-481, XP002029393 PICTON, S., ET AL.: "Altered fruit ripening and leaf senescence in tomatoes expressing an antisense ethylene-forming enzyme transgene" see the whole document --- -/--	2-4,7,8, 10

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
14 April 1997	25.04.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Maddox, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PC1/GB 96/03076

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NUCLEIC ACIDS RESEARCH, vol. 14, 1986, pages 5229-5239, XP002029394 KOES, R.E., ET AL.: "Floral tissue of Petunia hybrida (V30) expresses only one member of the chalcone synthase multigene family" see figure 3	6 13
X	GENE, vol. 81, 1989, pages 245-257, XP002029395 KOES, R.E, ET AL.: "Cloning and molecular characterization of the chalcone synthase multigene family of Petunia hybrida" see the whole document	6
A	WO 94 21794 A (ZENECA LTD ;ABU BAKAR UMI KALSOM (MY); BARTON SARAH LOUISE (GB); G) 29 September 1994 see page 25, last paragraph - page 26 see page 38 - page 45	1-4,7,8, 10
A	PLANT MOLECULAR BIOLOGY, vol. 19, 1992, pages 69-87, XP000571526 GRAY, J., ET AL.: "Molecular biology of fruit ripening and its manipulation with antisense genes" see page 82, left-hand column - page 83	2-4,7,8, 10
A	IN VITRO CELLULAR & DEVELOPMENTAL BIOLOGY PLANT, vol. 31, no. 1, January 1995, pages 36-43, XP002029396 MATHEWS, H., ET AL.: "GENETIC TRANSFORMATION OF STRAWBERRY: STABLE INTEGRATION OF A GENE TO CONTROL BIOSYNTHESIS OF ETHYLENE" see the whole document	9,11,12
A	WO 91 08299 A (ICI PLC) 13 June 1991 see claims 3,15	1-13
A	PLANT MOLECULAR BIOLOGY 27 (6). 1995. 1097-1108. , XP002029397 WILKINSON J Q ET AL: "Identification of mRNAs with enhanced expression in ripening strawberry fruit using polymerase chain reaction differential display." see the whole document	13

-/--

1

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 96/03076

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EMBL SEQUENCE DATABASE, REL. 34, 23-DEC-1992, ACCESSION NO. X69664, XP002029398 DAVIES, K.M.: "Malus sp. mRNA for naringenin, 2-oxoglutarate, 3-dioxygenase" see sequence	13
A	EMBL SEQUENCE DATABASE, REL. 35, 27-APR-1993, ACCESSION NO. X71360, XP002029399 DAVIES K.M.: "Malus sp. mRNA for anthocyanidin hydroxylase" see sequence	13
A	EMBL SEQUENCE DATABASE, REL. 38, 6-FEB-1994, ACCESSION NO. X77464, XP002029400 HUGHES, J., ET AL.: "M. esculenta Crantz CGT7 mRNA for UTP-glucose glucosyltransferase" see sequence	13
A	EMBL SEQUENCE DATABASE, REL. 40, 13-JUL-1994, ACCESSION NO. L33533, XP002029401 LIM, C.O., ET AL.: "Brassica rapa pekinensis EST F0166, partial mRNA" see sequence	13
A	EMBL SEQUENCE DATABASE, REL.37, 23-SEP-1993, ACCESSION NO. X74514, XP002029402 SCHWEBEL-DUGUE, N., ET AL.: "A.thaliana mRNA for beta-fructosidase" see sequence	13
A	EMBL SEQUENCE DATABASE, REL. 40, 13-JUL-1994, ACCESSION NO. Z35163, XP002029403 WEBER, H.: "V.faba VFCWINV2 mRNA for cell wall invertase II" see sequence	13

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 96/03076

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9306711 A	15-04-93	AU 2772792 A	03-05-93
WO 9214831 A	03-09-92	AU 1456292 A	15-09-92
		BR 9205480 A	01-03-94
		EP 0573566 A	15-12-93
		HU 66831 A	30-01-95
		US 5576428 A	19-11-96
WO 9421794 A	29-09-94	AU 6262394 A	11-10-94
		CA 2158473 A	29-09-94
		EP 0689594 A	03-01-96
		JP 8507923 T	27-08-96
WO 9108299 A	13-06-91	AT 140977 T	15-08-96
		AU 646422 B	24-02-94
		AU 6974591 A	26-06-91
		DE 69027996 D	05-09-96
		DE 69027996 T	09-01-97
		EP 0502995 A	16-09-92
		ES 2090304 T	16-10-96
		JP 5504056 T	01-07-93
		US 5413937 A	09-05-95
		US 5296376 A	22-03-94